Pathogenic Anti-Desmoglein 3 mAbs Cloned from a Paraneoplastic Pemphigus Patient by Phage Display

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Paraneoplastic pemphigus (PNP) is an autoimmune blistering disease associated with lymphoproliferative neoplasms and characterized by antibodies against plakins and desmoglein 3 (Dsg3). Anti-Dsg3 antibodies have a primary role in blister formation in PNP. In this study, we used phage display to clone monoclonal anti-Dsg3 antibodies from a PNP patient to further characterize their pathogenicity. We isolated 20 unique Dsg3-reactive mAbs, which we classified into four groups according to the heavy-chain complementarity-determining region 3 (CDR3) region. Genetic analyses demonstrated that three antibody groups used the VH1-46 gene (18 clones) and one group used the VH1-02 gene (2 clones). The results of an *in vitro* keratinocyte dissociation assay and a human skin organ culture injection assay showed that three antibodies displayed pathogenic activity in blister formation with different potencies. Epitope mapping using domain-swapped Dsg3/Dsg2 showed that these pathogenic mAbs bound Ca^{2+}-dependent conformational epitopes in the middle portion of the extracellular region of Dsg3 (EC2 and EC3 domains), in contrast to most previously characterized pathogenic pemphigus vulgaris antibodies, which bound to the EC1 domain of Dsg3. These mAbs reflect the unique polyclonal nature of anti-Dsg3 antibodies in PNP and represent an important tool for detailing the pathophysiological mechanisms of blister formation in PNP.

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INTRODUCTION

Pemphigus is a group of autoantibody-mediated blistering diseases of the skin and mucous membranes. Classic pemphigus includes two major subtypes: pemphigus vulgaris (PV) and pemphigus foliaceus (PF) (Stanley and Amagai, 2006). Autoimmune targets for these diseases are desmoglein (Dsg) 3 and Dsg 1, respectively. Dsg3 and Dsg1 are members of the cadherin family of cell–cell adhesion molecules localized in desmosomes (Amagai *et al.*, 1991; Stanley, 1993; Amagai, 1996).

Paraneoplastic pemphigus (PNP) is a unique subtype of pemphigus associated with underlying neoplasms (Anhalt *et al.*, 1990; Mehregan *et al.*, 1993). PNP is clinically characterized by the presence of intractable stomatitis and polymorphous skin eruptions. Histological findings include suprabasal acantholysis, keratinocyte necrosis, and vacuolar interface dermatitis (Anhalt *et al.*, 1990). PNP patients are known to possess characteristic autoantibodies against several members of the plakin family (desmoplakins I and II, BPAG1, envoplakin, periplakin, and plectin; Anhalt *et al.*, 1990; Oursler *et al.*, 1992; Hashimoto *et al.*, 1995; Kim *et al.*, 1997; Borradori *et al.*, 1998; Kiyokawa *et al.*, 1998; Mahoney *et al.*, 1998; Proby *et al.*, 1999), as well as autoantibodies against Dsg3, a common feature of PV (Joly *et al.*, 1994). Of these autoantibodies, those against Dsg3 are considered to have a primary pathogenic role in blister formation in PNP patients (Amagai *et al.*, 1998). Plakins are located in the cytoplasm of keratinocytes, and the circulating antibodies cannot access them. In a previous study, the removal of anti-Dsg3 IgG eliminated the pathogenic activity of PNP sera in passive transfer experiments in neonatal mice, and affinity-purified polyclonal anti-Dsg3 antibodies from PNP patient sera induced blister formation in neonatal mice (Amagai *et al.*, 1998).

Epitope analysis of PNP patient sera has revealed that Dsg3 epitopes are distributed more broadly on the extracellular domains of Dsg3 than those of PV patients (Futei *et al.*, 2003). This suggests that the autoimmune response against Dsg3 in PNP is more diversified than that in PV.

Phage display has been used to isolate anti-Dsg mAbs from PV and PF patients as single-chain variable fragments (scFvs). Pathogenic and nonpathogenic antibodies have been isolated (Payne *et al.*, 2005; Ishii *et al.*, 2008; Yamagami *et al.*, 2010).

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Abbreviations: CDR3, complementarity-determining region 3; Dsg3, desmoglein 3; ETA, exfoliative toxin A; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; ScFv, single-chain variable fragment

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Characterizations of these antibodies have revealed that an scFv form of anti-Dsg mAb can reproduce Dsg-binding specificities. Moreover, the pathogenic scFvs in these studies were capable of inactivating Dsgs and inducing blister formation. In addition, genetic analysis of the isolated antibodies revealed both heavy-chain and light-chain restriction, where the heavy chain was restricted to the VH1, VH3, and VH4 genes. The antibodies that bound to Dsg3 and/or Dsg1 were derived from different parental B-cell clones.

In this study, to better understand the pathophysiological mechanisms in PNP, we constructed a phage antibody library from PNP patient samples and isolated a cohort of anti-Dsg3 mAbs in the form of scFvs. We characterized the pathogenic activity of these isolated antibodies and characterized the corresponding conformational epitopes on Dsg3. In addition, we analyzed gene usage and the sequences of the variable regions and compared them with those of anti-Dsg3 antibodies isolated from PV patients. We isolated pathogenic anti-Dsg3 mAbs from a PNP patient. We found that the mAbs we isolated recognized the EC2 and EC3 domains of Dsg3, different pathogenic epitopes than those that are commonly recognized by anti-Dsg3 mAbs from PV patients.

RESULTS

Anti-Dsg3 mAbs isolated from a PNP patient shared VH genes with those from PV patients

We constructed a phage antibody library from peripheral blood lymphocytes from a PNP patient. The library was screened through multiple rounds of panning on immobilized baculovirus-produced Dsg3 using standard panning methods. First, we isolated 14 unique Dsg3-reactive monoclonal phage antibodies based on heavy- and light-chain nucleotide sequences. These clones were derived from only one heavy-chain gene (VH1-46) and used the same light-chain gene (2c). We then panned the library in the presence of previously isolated clones (PNP-A1 and PNP-B1) by epitope blocking (Ishii *et al.*, 2008), and isolated six additional clones encoded by VH1-46 and VH1-02 heavy-chain genes.

Pathogenic anti-Dsg3 mAbs isolated from PV patients use the VH1-46, VH1-69, VH3-07, and VH4b genes (Payne *et al.*, 2005; Yamagami *et al.*, 2010). Of the 20 isolated clones from the PNP patient, 18 shared VH1-46. Because the H-CDR3 (heavy-chain complementarity-determining region 3) is important for the pathogenicity of PV and PF antibodies (Yamagami *et al.*, 2010), we classified the 20 isolated clones into four subgroups (A, B, C, and D) according to their H-CDR3 sequences. Three groups (A, B, and C) were encoded by VH1-46 and one group (D) was encoded by VH1-02. Table 1 summarizes the genetic and immunological characterization of representative clones from each group. The nomenclature is as follows: PNP-A1 = paraneoplastic pemphigus, group A, clone number 1.

Three anti-Dsg3 mAbs bound to the surfaces of human keratinocytes

As expected, all clones bound to human Dsg3 ELISA. Immunofluorescence analysis of normal human skin showed that representative clones from the three groups encoding

Table 1. Anti-Dsg3 mAbs isolated from the PNP library

		Genetic characterization						Immunological characterization											
		Heavy chain				Light chain		ELISAs/IPIB					IIF		Epitope		Pathogenicity assays		
								Human			Mouse		Human	Mouse					
Clone name	Group	V gene	D gene	J gene	CDR3	V gene		Dsg3	Dsg1	Dsg2[1]	Dsg3[2]	Dsg1[2]	skin	skin/tail	Calcium dependency	Human Dsg3[1]	*In vitro* dissociation	Organ culture human skin	
PNP-A1	A	VH1-46	D6-13/DN1	JH4b	yycvregiaadgmdywgq	VL2c		+	–	–	–	–	+	–	+	EC3	+	+ (Focal)	
PNP-A2	A	VH1-46	D6-13/DN1	JH5b	yycvregiaadgmdywgq	VL2c		+	–	–	ND	ND	+	–	ND	EC3	ND	ND	
PNP-B1	B	VH1-46	D6-13/DN1	JH4b	yycardsipmmghdywgq	VL2c		+	–	–	+	–	+	Weak +	+	EC2	+	+ (Focal)	
PNP-B2	B	VH1-46	D6-13/DN1	JH4b	yycardsipmmghdywgq	VL2c		+	–	–	ND	ND	+	ND	+	EC2	ND	ND	
PNP-C1	C	VH1-46	D6-25	JH4b	yycargsglivpaayfdywgq	VL1g		+	–	–	–	–	+	–	+	EC2	++	+	
PNP-D1	D	VH1-02	D4	JH4b	yycasgvvvpaalddredywgq	VL2c		+	–	–	–	–	–	–	ND		–	–	

Abbreviations: CDR3, complementarity-determining region 3; Dsg, desmoglein; IIF, indirect immunofluorescence; IPIB, immunoprecipitation/immunoblotting; ND, not done; PNP, paraneoplastic pemphigus.
[1]Tested by IPIB.
[2]Tested by ELISA.

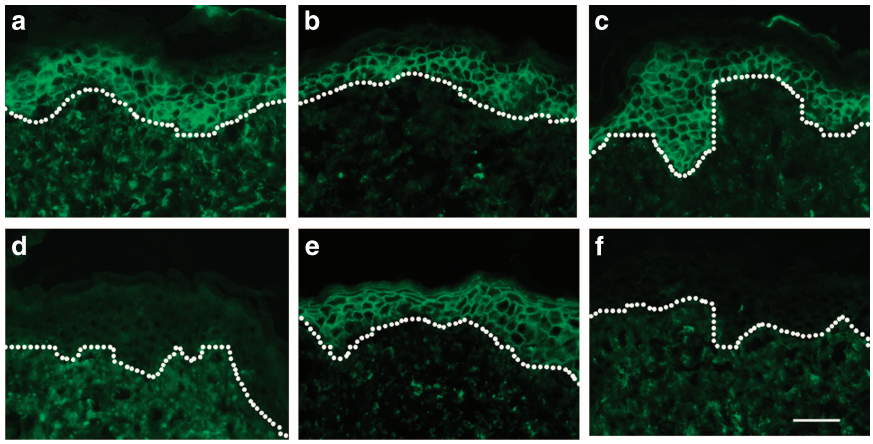

Figure 1. Immunological analysis of anti-desmoglein 3 (Dsg3) mAbs isolated from the paraneoplastic pemphigus (PNP) patient. Indirect immunofluorescence of anti-Dsg3 single-chain variable fragment (scFv) clones in human skin. (**a**) PNP-A1, (**b**) PNP-B1, and (**c**) PNP-C1showed cell surface staining of the keratinocytes, whereas clone (**d**) PNP-D1 was negative. (**e**) An scFv isolated from a pemphigus vulgaris (PV) patient (D31)2/28 was used as a positive control. (**f**) Pretreatment of human skin cryosections with EDTA prevented the cell binding of PNP-C1 to keratinocytes. Bar = 100 μm.

VH1-46 (PNP-A1, PNP-B1, and PNP-C1) were bound to the surfaces of keratinocytes. A representative clone from the VH1-02 group (PNP-D1) did not bind to cell surfaces (Figure 1), although the antibody bound to recombinant Dsg3 ELISA and was detected by immunoprecipitation/immunoblotting (data not shown).

We tested reactivity in mice by indirect immunofluorescence and ELISA. Only PNP-B1 showed weak cell surface staining of mice keratinocytes by indirect immunofluorescence and bound to mouse Dsg3 ELISA. None of the antibodies bound to mouse Dsg1 ELISA (Table 1). Because three antibodies did not crossreact to mice, we could not use mice to test *in vivo* pathogenicity.

Two anti-Dsg3 mAbs bound to the EC2 domain of Dsg3 and one mAb bound to the EC3 domain through calcium-sensitive epitopes

As most pathogenic autoantibodies from PV and PF bind to calcium-sensitive epitopes (Amagai *et al.*, 1995; Payne *et al.*, 2005; Ishii *et al.*, 2008), we analyzed whether the anti-Dsg3 mAbs from the PNP patient would bind calcium-sensitive epitopes. The cell surface binding of keratinocytes by PNP-A1, PNP-B1, and PNP-C1 was abrogated when the human skin was preincubated with EDTA (Figure 1f). This suggests that the clones bound to calcium-dependent conformational epitopes.

We further characterized the Dsg3 epitopes recognized by the isolated antibodies by immunoprecipitation/immunoblotting using a series of Dsg3/Dsg2 domain-swapped molecules produced using a baculovirus expression system (Ohyama *et al.*, 2012). PNP-A1 bound to the EC3 domain of Dsg3, whereas PNP-B1 and PNP-C1 bound to the EC2 domain (Figure 2b). We also characterized the epitopes recognized by the polyclonal Dsg3 antibodies from the PNP patient. The epitopes were broad, and covered EC1 to EC4 (Figure 2b). This contrasts with classic PV patients, in whom the dominant epitopes recognized by Dsg3 antibodies are EC1 and EC2 (Figure 2a and b).

The isolated mAbs represent only a subset of the polyclonal anti-Dsg3 antibodies in the PNP patient

The isolated mAbs were tested for their ability to inhibit the binding of polyclonal antibodies in the PNP patient's serum to human Dsg3 ELISA. Each scFv competed with the PNP patient's serum in the ELISA plate in serial dilutions. PNP-A1, PNP-B1, PNP-C1, and PNP-D1 inhibited at most only 15% (± 2.4), 11% (±3.2), 9% (±4.3), and 13% (±5.2), respectively (Figure 2c), of the binding of the patient's serum to Dsg3. We further prepared a mixture of equal volumes of the four isolated scFvs and then competed this mixture in serial dilutions with the patient's serum on the ELISA plate. The mixture inhibited binding of polyclonal antibodies in the patient's serum by only 16% (±1.3). This suggests that the isolated mAbs represent only a subset of the polyclonal anti-Dsg3 antibodies in the PNP patient's serum and that the majority of polyclonal anti-Dsg3 antibodies may recognize different epitopes.

Three anti-Dsg3 mAbs caused fragmentation of epidermal sheets in an *in vitro* dissociation assay

To examine the pathogenicity of the mAbs, we performed an *in vitro* dissociation assay using normal human epidermal keratinocytes (Ishii *et al.*, 2005). The normal human epidermal keratinocytes were incubated with the antibodies (1 μg ml^{-1}) in the presence of 0.5 μg ml^{-1} exfoliative toxin A (ETA), which inhibits Dsg1 (Amagai *et al.*, 2000; Hanakawa *et al.*, 2004; Nishifuji *et al.*, 2010). Gentle pipetting will result in fragmentation of the epidermal cell sheet if the antibody is pathogenic. AK23 (IgG form) was used as a positive control (Figure 3). AK23 is a well-characterized mouse bivalent anti-Dsg3 mAb that crossreacts with human Dsg3 (Tsunoda *et al.*, 2003). ETA alone was used as a negative control (Figure 3). The dissociation scores for PNP-A1, PNP-B1, and PNP-C1 were 10 (±0.09), 9 (±0.9), and 32 (±10), respectively ($n = 3$). We also prepared mixtures combining two scFvs (PNP-A1 + PNP-B1, PNP-B1 + PNP-C1 and PNP-A1 + PNP-C1) and all the three scFvs, of which the final concentration

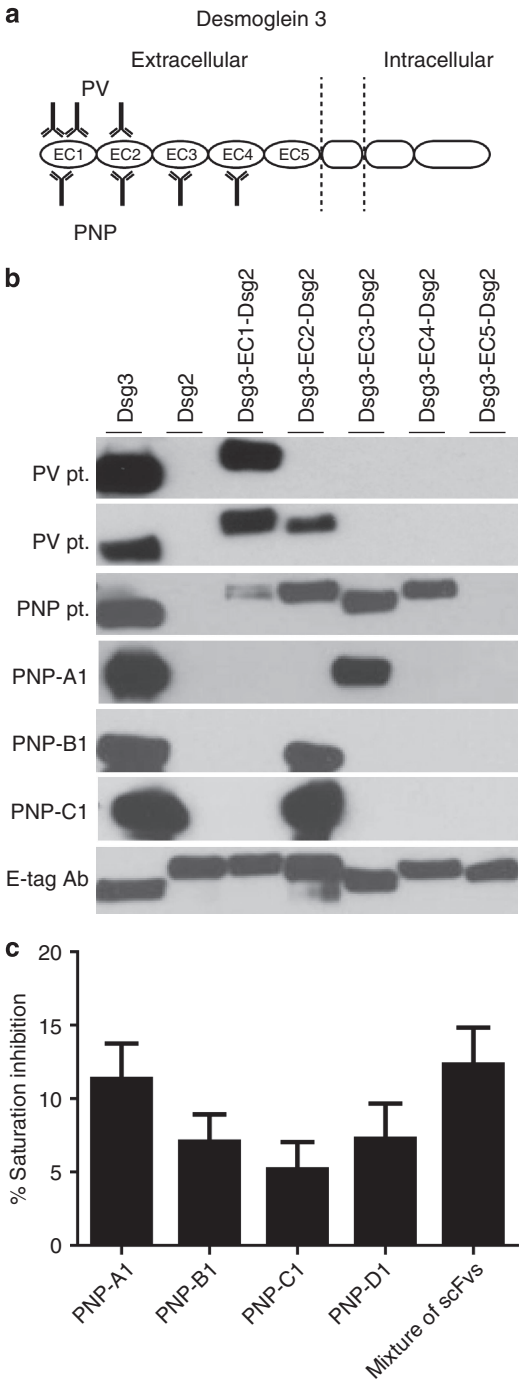

Figure 2. The isolated antibodies bound to the EC2 and EC3 domains of desmoglein 3 (Dsg3) and accounted for some of the polyclonal antibodies in the paraneoplastic pemphigus (PNP) patient (pt.). (**a**) Schematic diagram for the binding of the anti-Dsg3 antibodies in PNP and pemphigus vulgaris (PV) patients. (**b**) Epitope mapping of the patient's serum and the isolated single-chain variable fragments (scFvs). Immunoprecipitation/immunoblot analysis of PV, PNP patients' sera, the isolated scFvs, and anti-E-tag antibody (positive control) was performed using recombinant human Dsg3/Dsg2 domain-swapped molecules. The PNP patient's Dsg3 epitopes were EC1 (weak), EC2, EC3, and EC4. In classic PV patients, the epitopes are mainly EC1 and EC2. (**c**) Competition ELISA of the four mAbs isolated from the PNP patient's serum in a human Dsg3-coated ELISA plate. Data are shown as the mean ± SD.

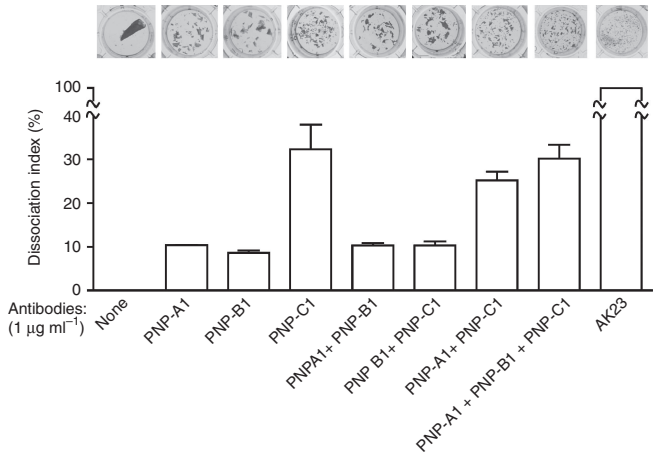

Figure 3. Anti-desmoglein 3 (Dsg3) single-chain variable fragments (scFvs) caused the dissociation of cultured human epidermal keratinocytes. Cultured human keratinocytes were incubated with exfoliative toxin A (ETA) with or without scFvs or a mixture of the scFvs at a concentration of 1 μg ml^{-1} and then treated with dispase to release cell monolayers. Released cell sheets were exposed to mechanical shear stress to evaluate intercellular adhesion. The dissociation index was calculated from the number of cell sheet fragments (*N*) for each treatment using the following formula: ((*N* with mAb–*N* with ETA only)/(*N* with AK23–*N* with ETA only)) × 100. Data are shown as the mean ± SD. Top panel shows the photos of the wells. PNP, paraneoplastic pemphigus.

of the total scFvs was 1 μg ml^{-1}. The dissociation scores of PNP-A1 + PNP-B1, PNP-B1 + PNP-C1, and PNP-B1 + PNP-C1 were 10 (± 1), 10 (± 2), and 25 (± 3), respectively. The dissociation score of the mixture of the three scFvs was 30 (± 6). None of the combined scFvs showed higher dissociation indexes than the single scFvs, indicating that there is no apparent synergism among the scFvs examined in this study.

Three anti-Dsg3 mAbs caused acantholysis in organ-cultured human skin

Two scFvs failed to crossreact with mice, and thus neonatal mice could not be used in an *in vivo* assay. Thus, we assessed pathogenicity using human skin tissues. The scFvs were injected into the dermis of normal human skin in organ culture. The antibodies were injected with a minimal amount of ETA to inactivate Dsg1. An scFv isolated from a PV patient ((D31)2/28; Payne *et al.*, 2005) was used as a positive control. (D31)2/28 crossreacts with both Dsg3 and Dsg1, and thus ETA is not required to inactivate Dsg1. Direct immunofluorescence analysis of the skin organ cultures showed binding of PNP-A1, PNP-B1, and PNP-C1 to the surfaces of keratinocytes (data not shown). Hematoxylin and eosin staining showed that PNP-C1 caused typical suprabasal acantholysis, indicating that the antibody is pathogenic (Figure 4). PNP-C1 showed pathogenic activity in a dose-dependent manner when 50, 100, or 150 μl scFv (1 μg μl^{-1}) was injected. PNP-A1 and PNP-B1 only produced focal acantholysis when 100 or 150 μl scFv (1 μg μl^{-1}) was injected. We quantitatively evaluated acantholysis by comparing the width of the blister with the width of the whole skin specimen, a measure we called the organ culture acantholysis index (Figure 4). PNP-A1,

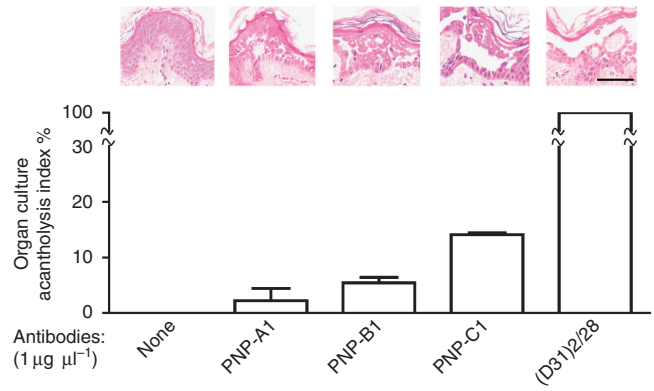

Figure 4. Pathogenicity assay (injection of single-chain variable fragments (scFvs) into organ cultured human skin). Injection of PNP-C1 (paraneoplastic pemphigus, group C, clone number 1) with low-dose exfoliative toxin A (ETA) caused suprabasal acantholysis. PNP-A1 and PNP-B1 caused focal acantholysis. An scFv isolated from a pemphigus vulgaris (PV) patient (D31)2/28 was used as a positive control. We did not add ETA to the positive control because the positive control inhibits both desmoglein (Dsg)3 and Dsg1 (Payne *et al.*, 2005). Calculation of the degree of acantholysis based on the organ culture acantholysis index (OCAI). Hematoxylin and eosin staining was performed, and the OCAI was calculated after the injection of 150 μl of 1 μg μl^{-1} scFv. Data are shown as the mean ± SD. Bar = 50 μm.

PNP-B1, and PNP-C1 scored 2.2 (±3.1), 5.4 (±0.9), and 14.1 (±0.5), respectively, when 150 μl scFv (1 μg μl^{-1}) was injected.

The findings of the *in vitro* dissociation assay and organ culture assay together suggest that PNP-A1, PNP-B1, and PNP-C1 have the ability to induce loss of cell adhesion. Moreover, the lower number of fragments in the dissociation assay and the focal acantholysis in the skin organ culture suggest that PNP-A1 and PNP-B1 are weaker than PNP-C1. PNP-D1 caused neither fragmentation of cell sheets nor blistering in organ culture (data not shown), which suggests that it is not pathogenic.

DISCUSSION

Phage display has previously been used to isolate mAbs from PV (Payne *et al.*, 2005) and PF patients (Ishii *et al.*, 2008; Yamagami *et al.*, 2010). In this study, we constructed a phage library from a PNP patient and succeeded in isolating a cohort of anti-Dsg3 mAbs. We proved that three anti-Dsg3 mAbs (PNP-A1, PNP-B1, and PNP-C1) possess the ability, as scFvs, to induce blister formation in the skin. The ability of the isolated antibodies to induce loss of cell adhesion was weaker than that of the positive controls, as assessed by the number of fragments in a dissociation assay and the extent of acantholysis in an organ culture injection assay.

The differences in the abilities of the scFvs isolated from the PNP patient in this study and previously isolated antibodies from PV patients to induce loss of cell adhesion may be explained by the epitopes of the antibodies. Whereas PNP-B1 and PNP-C1 bound to the EC2 domain of Dsg3, PNP-A1 bound to EC3. This result contrasts with the previous finding that pathogenic mAbs from PV patients bound to the EC1 region of Dsg3, which is predicted to form the intercellular trans-adhesive interface (Tsunoda *et al.*, 2003; Payne *et al.*, 2005). This is the region containing the dominant epitopes in human PV. In a previous study, mouse mAbs were isolated from PV model mice generated by transfer of naive Dsg3$^{-/-}$ splenocytes (Kawasaki *et al.*, 2006). Antibodies recognizing the middle portion of Dsg3 proved to be weakly pathogenic. Taken together with our data, these findings suggest that anti-Dsg IgG antibodies that recognize the middle and C-terminal extracellular domains of Dsg3 may also contribute to blister formation. Moreover, our findings indicate that antibodies against the EC2 and EC3 domains of Dsg3 are involved in blister formation.

It was previously reported that a combination of weakly pathogenic anti-Dsg3 IgG autoantibodies had synergistic pathogenic effects on blister formation (Kawasaki *et al.*, 2006). When the pathophysiology of PNP is considered, the severe phenotype may be caused by the synergistic effects of low-potency pathogenic anti-Dsg3 antibodies that recognize various epitopes. However, the combination of the three isolated mAbs did not show any apparent synergistic effect in dissociation assay (Figure 3). Other anti-Dsg3 mAbs may be required to show synergistic effect on keratinocyte dissociation. It will be interesting to study whether pathogenic antibodies from PNP and PV patients differ in their activation of signal transduction pathways. In addition, it may be interesting to study whether the binding of PNP antibodies to Dsg3 for long periods induces secondary inflammation in the skin, a phenomenon that may be observed in PNP.

We analyzed the gene usage of the isolated Dsg3 mAbs. Three groups of mAbs shared the VH1-46 locus, and one group was derived from VH1-02. Some mAbs isolated from PV patients by phage display use the same VH1-46 gene (Payne *et al.*, 2007; Yamagami *et al.*, 2010). In one study using the heterohybridoma technique, anti-Dsg3 and anti-Dsg1 antibodies from PV patients did not use VH1-46 (Qian *et al.*, 2007). In another study whose aim was to clone B cells isolated by flow cytometry from peripheral blood of PV patients, some Dsg3-specific B cells used VH1-46 (Yamagami *et al.*, 2008). This suggests that Dsg3-specific B cells from PNP and PV patients may come from restricted parental B cells.

In conclusion, we isolated a cohort of Dsg3 mAbs from a PNP patient, three of which had the ability to induce blister formation. The pathogenic mAbs recognized the middle portion of Dsg3 (the EC2 and EC3 domains), in contrast to the pathogenic PV antibodies. These findings reflect the unique polyclonal nature of anti-Dsg3 IgG antibodies in PNP, and indicate key differences between PNP and PV in terms of the pathophysiological mechanisms of blister formation and autoimmune responses. The antibodies we have isolated will be useful for elucidating the molecular mechanisms of blister formation in PNP and the adhesive function of Dsgs.

MATERIALS AND METHODS

PNP patient

We used peripheral blood from a 76-year-old male patient with active PNP to construct the PNP library (Figure 5). The patient was diagnosed with B-cell lymphoma through microscopic examination

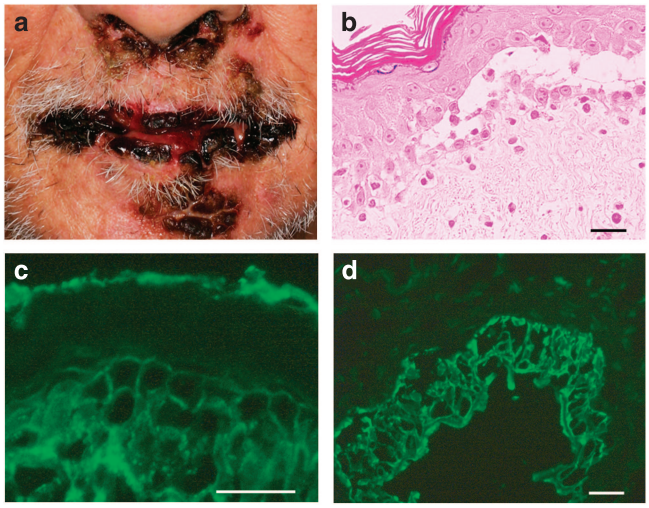

Figure 5. Clinical, histological, and immunofluorescence photographs of the paraneoplastic pemphigus (PNP) patient from whom the phage antibody library was constructed. (**a**) The patient suffered from severe mucosal and skin erosions. (**b**) Hematoxylin and eosin staining of the patient's skin biopsy showed suprabasal blistering and vacuolar degeneration. (**c**) Direct immunofluorescence (DIF) of the patient's skin showed IgG deposition on the surfaces of keratinocytes. (**d**) Indirect immunofluorescence of the patient's IgG-stained rat bladder transitional epithelium. Bars = 100 μm.

of a lymph node biopsy. This study was approved by the institution review board of Keio University and was conducted according to the Declaration of Helsinki Principles. All samples were used with informed consent.

Construction of the phage library

Using previously described methods (Barbas, 2001), we constructed separate IgG-κ and IgG-λ phage libraries from 1×10^7 mononuclear cells isolated from 50 ml peripheral blood collected from the PNP patient. Reverse transcriptase–PCR was used to amplify the Ig variable regions of the heavy (V_H) and light chains (V_L). The gene fragments were then cloned into the phagemid vector pComb3X (Scripps Institute, La Jolla, CA). The phagemid library was electroporated into the XL-1 Blue strain of *E. coli* (Stratagene, La Jolla, CA) through superinfection by the helper phage VCSM13 (Stratagene). In this system, filamentous phage particles express scFv antibodies (with a C-terminal $(His)_6$ tag and an HA tag) fused to the pIII bacteriophage coat protein. Recombinant phages were purified from culture supernatants by polyethylene glycol precipitation and resuspended in phosphate-buffered saline (pH 7.4) containing 1% BSA and 1 mM $CaCl_2$. The library comprised more than 2×10^8 independent transformants, as determined by titering on XL1-Blue *E. coli* after transformation. To validate library diversity, we analyzed the sequences of 11 phage clones from the unpanned library. The results showed marked heterogeneity in V_H and V_L gene usage.

Panning of the phage library

ELISA plates coated with recombinant Dsg3 (MBL, Nagoya, Japan) were used to isolate phage clones as described previously (Payne *et al.*, 2005). Briefly, four wells were incubated with blocking buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, and 3% skim milk) at room temperature for 1 hour. The phage library was diluted into blocking buffer and then incubated in Dsg3-coated wells for 2 hours at room temperature. After five washes with Tris-buffered saline/Ca containing 0.1% Tween-20, adherent phages were eluted with 76 mM citric acid (pH 2.0) and then neutralized with 2 M unbuffered Tris. The eluted phages were amplified in XL1-Blue *E. coli* and rescued by superinfection with VCSM13. Phages were harvested from bacterial culture supernatants and then repanned four more times. Individual phage clones were isolated at each round of panning and then analyzed for binding to Dsg3 by ELISA using a horseradish peroxidase–conjugated anti-M13 antibody (GE Healthcare Bio-Sciences, Uppsala, Sweden). For epitope-blocked panning, the phage library was first mixed with the purified recombinant scFv clones PNP-A1 and PNP-B1, and then incubated on immobilized Dsg3 for 2 hours at room temperature.

Sequence analysis of scFv antibodies

Recombinant phagemids were purified using a plasmid preparation system (Qiagen Sciences, Germantown, MD), and the V_H and V_L inserts were sequenced using pComb3X-specific primers as described previously (Barbas, 2001). The nucleotide sequences were compared with germline sequences in the V Base sequence directory (http://vbase.mrc-cpe.cam.ac.uk/) to determine their germline gene origins and interrelatedness.

Production and purification of scFv antibodies

The Top10 F′ nonsuppressor strain of *E. coli* (Invitrogen, Carlsbad, CA) was infected with an individual phage clone, and soluble scFvs were purified from the bacterial periplasmic space using FastBreak (Promega, Madison, WI) and TALON Metal Affinity Resin (Clontech Laboratories, Mountain View, CA). The purity of the scFv preparation was evaluated by SDS-PAGE, followed by Coomassie staining, which showed a single predominant band.

Dsg3 scFv ELISA

The reactivity of scFvs against human Dsg3 was measured by Dsg3 ELISA using a horseradish peroxidase–conjugated anti-HA mAb (3F10; dilution, 1:1,000; Roche Diagnostics, Mannheim, Germany).

Direct and indirect immunofluorescence

Immunofluorescence for scFvs was performed on human skin and neonatal mouse skin and tail. Binding was detected through staining with rat anti-HA mAb (3F10; dilution, 1:100; Roche Diagnostics), followed by an Alexa Flour 488-conjugated anti-rat IgG (dilution, 1:200; Invitrogen).

Dissociation assay

Normal human epidermal keratinocytes (a gift from Ehime University) were grown on collagen-coated 12-well microplates (Iwaki Science Products, Tokyo, Japan) until confluent. Thereafter, 1.2 mM $CaCl_2$ and scFvs (1 μg ml^{-1}) were added, and the plates were incubated for 12 hours at 37 °C. ETA (0.5 μg ml^{-1}) was added and incubated for 2 hours. The cells were washed twice with phosphate-buffered saline (+), resuspended in 1 ml phosphate-buffered saline (+), and pipetted five times using a 1-ml pipette. The cells were fixed by adding formaldehyde (5%). A few drops of 0.02% crystal violet were added to stain the particles, with the plates being incubated overnight to allow the stain to be absorbed. Three pictures were taken and were analyzed manually. The dissociation index was

calculated from the number of fragments (*N*) using the following formula: ((*N* with mAb–*N* with ETA only)/(*N* with AK23–*N* with ETA only)) × 100. For the combination of scFvs, we prepared mixtures containing equal concentrations of two scFvs (PNP-A1 + PNP-B1, PNP-B1 + PNP-C1, and PNP-A1 + PNP-C1) or all of the three scFvs, whose final concentration of the total scFvs was 1 μg ml^{-1}.

Epitope mapping by immunoprecipitation/immunoblotting

To determine the epitopes of the PNP patient, as well as the scFvs, we used the following baculovirus-produced recombinant human Dsg3/Dsg2 domain-swapped molecules tagged with an E-tag and a histidine tag: Dsg3-EC1 (1-101), Dsg3-EC2 (95-221), Dsg3-EC3 (212-328), Dsg3-EC4 (320-440), and Dsg3-EC5 (440-551; Ohyama *et al.*, 2012). Baculovirus-infected insect cell culture supernatants containing recombinant molecules were incubated with the serum or scFvs for 30 minutes, and then immunoprecipitated with protein G Sepharose 4 Fast Flow beads (GE Healthcare Bio-Sciences) for serum or anti-HA agarose (Sigma-Aldrich, St Louis, MO) for scFvs (at 4 °C overnight with gentle rotation). After washing with Tris-buffered saline/Ca, immunoprecipitates were resuspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed with a horseradish peroxidase–conjugated anti-E-tag antibody (dilution, 1:5,000; GE Healthcare Bio-Sciences). Anti E-tag antibody (GE Healthcare Bio-Sciences) was used as positive control.

Human organ culture injection

Specimens of normal skin that were left over after skin biopsies were obtained from our dermatology outpatient clinic. After fat removal, the specimens were cut into pieces with a diameter of 4 mm. Intradermal injection of 100 μl purified scFv and low-dose ETA was then performed using an insulin syringe, and the specimens were transferred to Transwells (Corning, NY) containing MCDB medium (a gift from Ehime University) and 1.2 mM $CaCl_2$. At 24 hours, the skin was harvested for direct immunofluorescence and histology. The organ culture acantholysis index was calculated as (width of blister/total width of specimen) × 100.

Inhibition ELISA

Inhibition ELISA was performed as described previously (Ishii *et al.*, 2008). The diluted PNP serum competed with an scFv mixture in a Dsg3 ELISA, and was detected using horseradish peroxidase–conjugated anti-human IgG (MBL). The following calculation was used: % inhibition = (1 − [(OD PNP serum with competitors − OD AM3-13)/(OD PNP serum without competitors − OD AM3-13)]) × 100, where AM3-13 is an scFv isolated from a thrompocytopenic purpura patient.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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